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ORIGINAL PAPER

Keratinocytes seeded fibrin micro-carriers reconstitute an epidermis in full thickness wounds

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Abstract Following the advantages of single cell transplantation in comparison to sheet grafts we improved a spinning culture system for keratinocytes on completely biodegradable micro-carriers. Since previous in vivo investigations with different carrier materials (Cytodex® and PLGA) [Voigt et al. Tissue Eng 8(2):263–272, 2002; Voigt et al. Tissue Eng 5:563–572, 1999] revealed severe cellular inflammatory reactions and persistent remnants of micro-carriers in wounds, the aim of this study was to find a completely biodegradable, physiological and non-toxic material for new micro-carriers. We succeeded in setting up a keratinocyte spinning culture system using fibrin carriers. We were able to reconstitute the epidermis in full thickness wounds in an athymic nude mouse model and in an immunocompetent porcine wound model.

Keywords Fibrin micro-carriers · Full thickness wounds · Spinning culture system

Introduction

As it was shown earlier, it is possible to culture and transplant keratinocytes on a mobile carrier system and reconstitute a neoepithelium on a nude mouse full thickness wound model [6, 11, 12]. The advantage of this system is the possibility to expand the cells on the large total surface of the spherical carriers very quickly and to avoid further enzymatical steps to harvest the cells from a culture flask. The major drawback of the system is the significant inflammation reaction in the animal model due to the non-resorbable carrier material (Cytodex 3®), which consists of dextran coated with denaturated collagen [6, 12] and pH changes and without total resorption due to an improved carrier material polylactid acid (PLA) [5]. Further, the previous experiments were carried out in an immunoincompetent athymic mouse model. We advanced our spinning culture model with specially fabricated fibrin carrier in an immunocompetent porcine wound model. Fibrin is a proven and advantageous vehicle material for keratinocytes since fibrin matrix adhesion, proliferation and migration of keratinocytes facilitate via a transient overexpression of the fibronectin receptor [8–10]. Fibrin glue is anticipated to be a very useful vehicle and culture material as we know from several investigations and the clinical use of keratinocyte fibrin glue suspension (KFGS) [9, 10]. In this study we are able to show the feasibility of fabricated fibrin carrier to serve as cell culture model and transplantation vehicle. We also investigated the tissue compatibility of the fibrin carrier in the athymic mouse model and in an immunocompetent porcine wound model.

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Materials and methods

In vitro experiments

Fibrin carriers were inoculated with keratinocyte single cell suspension using the spinning culture technique.

Fibrin carriers were produced from lyophilized fibrin glue flakes which were crushed into micro-carrier pieces ranging between 50 and 500 μm in diameter (Baxter, Vienna, Austria).

In vitro Isolated human/porcine keratinocytes from secondary serum-free, subconfluent culture were inoculated directly with fibrin micro-carriers using the spinning culture technique (Fig. 1). Cell growth and proliferation were determined by counting and alamarBlue™ test [1]. Propidium–iodide staining was used to stain the cell nuclei and simplify counting.

Primary cell culture

A skin biopsy from a 35 year old patient undergoing plastic surgery was obtained and human keratinocyte cells were enzymatically harvested and cultured under serum-free conditions according to Boyce et al. [3] After reaching subconfluence, the cells were detached enzymatically from the culture flasks. A pig skin biopsy was taken from the same animal, which was later used for the autologous porcine wound model.

Micro-carrier

The fibrin carriers were produced from flakes of lyophilized fibrin glue flakes which were crushed into micro-carrier particles ranging between 50 and 500 μm in diameter (Baxter, Vienna, Austria). These microparticles could be inoculated directly with isolated human keratinocytes from secondary culture using the spinning culture technique.

Proliferation assay: alamarBlue™

The alamar Blue™ Assay (DAL 1100, Biosource international) is a water soluble fluoro/spectrometric indicator for cell growth, which relies on the metabolic activity of the cells.

Fig. 1 Spinning device in incubator

Spinner system

The spinner culture device consists of 500 ml spinner flasks with metal containing stirring arms and a unit of four electromagnetic plates fitting into a 37°C incubator (Integra Biosiences, Fernwald, Germany). This unit is driven by a computer system allowing adjustment of the rotating angle and the rotating speed (Fig. 1).

Spinner culture

Cells obtained from the flasks were inoculated with Fibrin micro-carriers in serum-free medium for 5 days in the spinner system. We used the culture technique as previously described by Voigt et al. [11, 12] for human and porcine keratinocytes. In detail we investigated the optimal parameters for evenly suspended particles. To keep the carrier in suspension a spinning continuous speed of 30 RPM was optimal. The rotation direction was chanced from clockwise to counter-clockwise after a rotation angle of 1260°.

In vivo experiments

Cell seeded carrier transplantation nude mouse model

Grafting was investigated utilizing a standard athymic nude mouse model, as previously described by Voigt et al. [12]. Full thickness wounds (2×2 cm) were created on the back of nude mice. Wounds were dressed with Biobrane® (Dow B. Hickam, Inc., Sugar Land, TX, USA) and sterile cotton gauze. Wound dressing was changed every day, biopsies taken at day 7, 14 and 21 postoperatively.

Experiments included cell seeded fibrin carriers, cell-free controls and human keratinocytes in fibrin resuspended (keratinocyte fibrin glue suspension — KFGS).

Wounds were transplanted with either:

- Group M I: fibrin carrier seeded with cells (n=12)
- Group M II: fibrin carrier without cells (n=12)
- Group M III: KFGS (n=12)

Porcine model

After 5 days in spinning culture the fibrin micro-carriers with autologous porcine keratinocytes were transplanted onto full thickness wounds. A margin of 1–1.5 cm was left as a barrier to the wound edge to prevent keratinocytes overgrowth from the wound's edges. Fig. 2 shows the transplantation of the single cell suspension to full thickness wounds.

We created in our standard porcine model, as previously described by Bannasch et al. [2] eight 3×3 cm full thickness

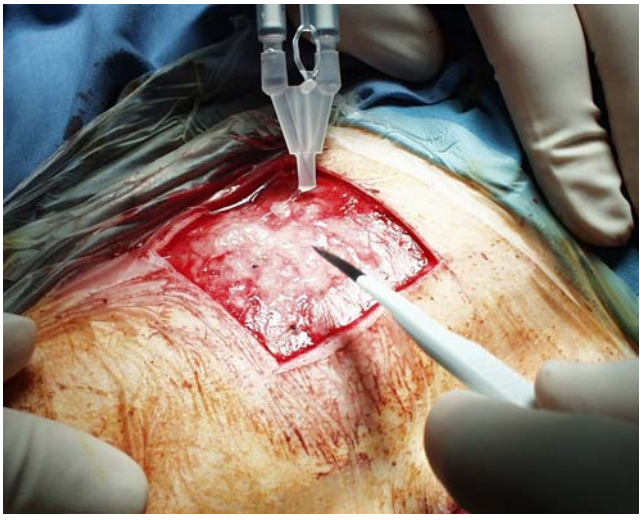

Fig. 2 Transplant procedure in pig model. The cell seeded fibrin carriers are transferred to the wound suspended in fibrin glue

wounds on the dorsum of four pigs. Wounds were transplanted either with:

- Group P I: fibrin carriers+autologous porcine keratinocytes (n=4) or:
- Group P II: keratinocyte fibrin glue suspension (KFGS) (n=4)

Keratinocyte density in group I was $0.08\times106/cm^2$ and in group II $0.75\times106/cm^2$. Transplanted wounds were dressed with Biobrane® (Dow B. Hickam, Inc., Sugar Land, TX, USA) and sterile cotton gauze. Wounds were observed for 21 days postoperatively.

Histology

Complete excision biopsies were taken on day 7, 14 and 21 postoperatively and stained with hematoxiline–eosine for microscopic investigations.

Direct immunofluorescence staining for MHC Class I (FITC, Sigma) was performed to prove the human origin of the epithelium in the nude mouse in vitro investigations.

Results

In vitro experiments

The cells were inoculated to the carrier suspension, the viable cells attached to the carrier after 2 to 4 h (Fig. 3). At that time cells started to grow on the carrier surface as seen in light microscopy. The shortest seeding time (4 h) was seen in the above mentioned parameters. Cells remained attached stably to the cells without leaving the carriers. Maximal proliferative capacity was shown at day 5 after inoculation in the spinning culture system (day of transplantation). At that day, shown in Fig. 4, we obtained double the amount of human keratinocytes compared to standard culture methods.

Proliferation assay: alamarBlue™

As shown in Fig. 5, the metabolic activity and proliferation of human keratinocytes on fibrin micro-carriers showed statistically significant cell growth with the maximum being on day 4.

In vivo experiments

Athymic mouse model

Histology In group M I, 7 days after transplantation, we could detect an orientation of epithelial cells to the surface of the wound and on the 14th day a complete epithelialisation of the wound (Fig. 6). No inflammatory cells were seen at the site of the carrier material on day 21. In the control group M II, epithelial ingrowth at the wound border on day 21 was detected (Fig. 7), there were no epithelium at days 7 and 14. The KFGS group (group M III) showed at day 7 no epithelium but some epithelial cells orientating to the wound surface, on day 14 a thin, closed epithelial layer and murine epithelial ingrowth at the wound border.

Immune staining Anti-MHC Class I immune staining showed human cells in the neoepithelium and surrounding the carriers 14 days after transplantation in group M I and M III (Fig. 8). The control wounds treated with carriers without cells seeded showed a thin layer of mouse epithelium, which did not bind the FITC antibody.

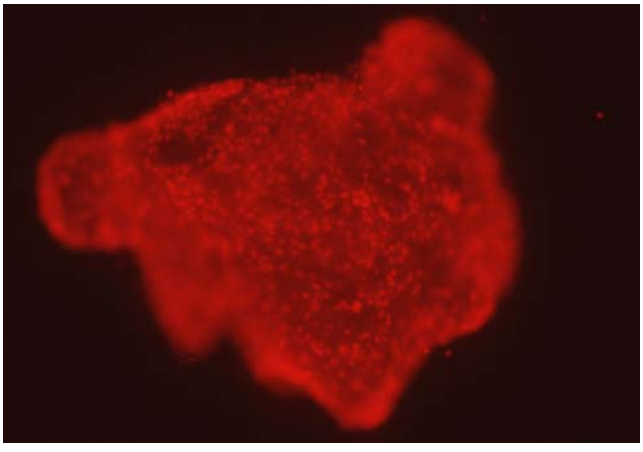

Fig. 3 4 h after inoculation of cells into the spinner culture. The seeded cells are highlighted by propidium–iodide staining

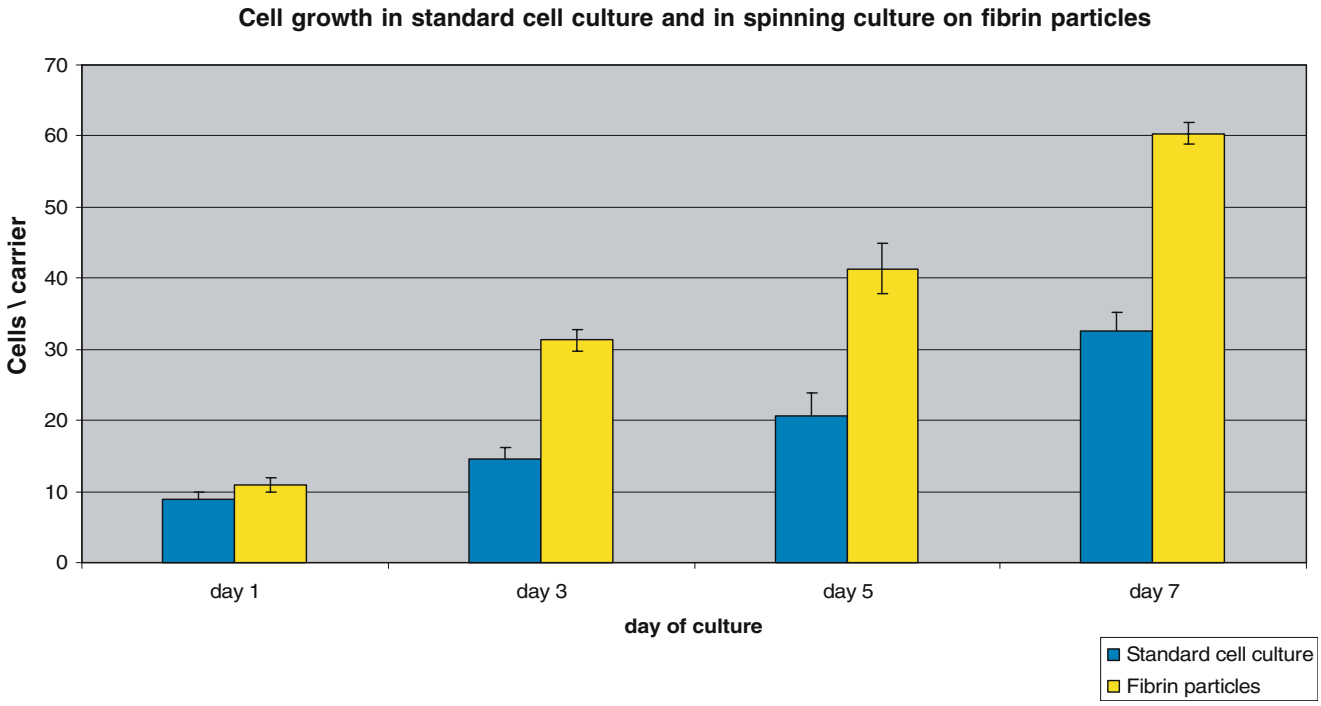

Fig. 4 Growth rate of keratinocytes in standard flask culture and in spinning culture on fibrin micro-carriers. On day 7 the cell gain is almost double in the spinning culture compared to the standard flask culture

Porcine model

No infection was observed during the whole experiment.

Histological staining showed on day 14 completely epithelialized transplantation-islands in wounds transplanted with autologous porcine keratinocytes on fibrin carriers. Peripheral areas not epithelialized indicate that epithelialization originated from transplanted cells. As shown in Fig. 9 a characteristic rete ridges formation has been developed with intra-epidermal cyst formation with vacuoles in former carrier locations. There is lysis of fibrin carriers with no inflammatory tissue-reaction around fibrin carriers.

Discussion

Fibrin micro-carriers in the spinning culture technique have been shown to be suitable to reconstitute an epidermis on full thickness wounds with either human or porcine keratinocytes.

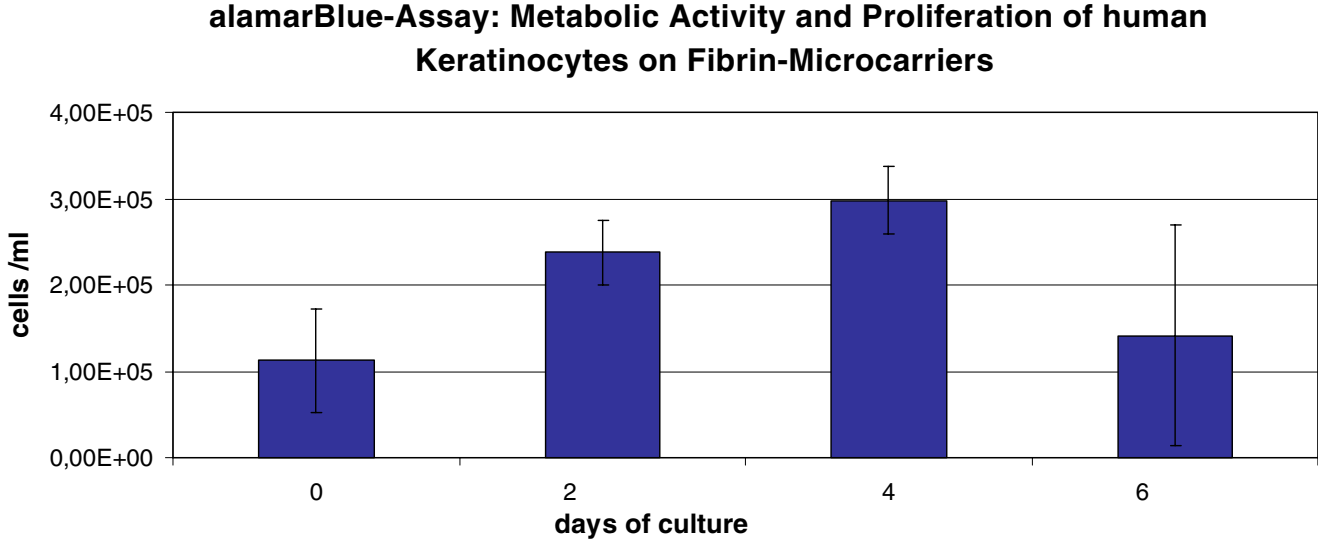

Fig. 5 alamar Blue assay showing a significantly enhanced metabolic activity and metabolism of seeded keratinocytes with maximum on day 4 ($p<0.001$)

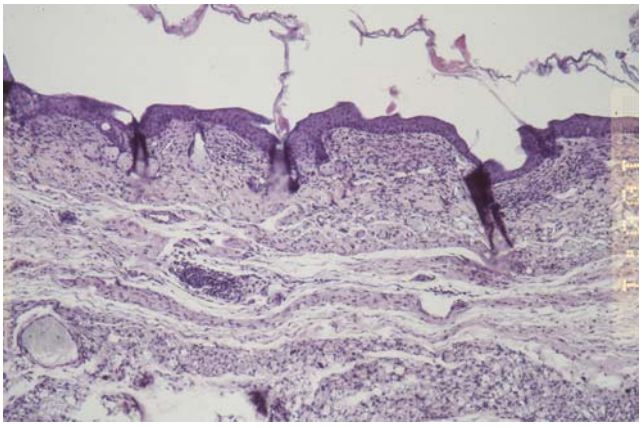

Fig. 6 HE staining of wound necropsy 14 days after transplantation of keratinocytes seeded micro-carriers. The epithelium is closed and keratinized

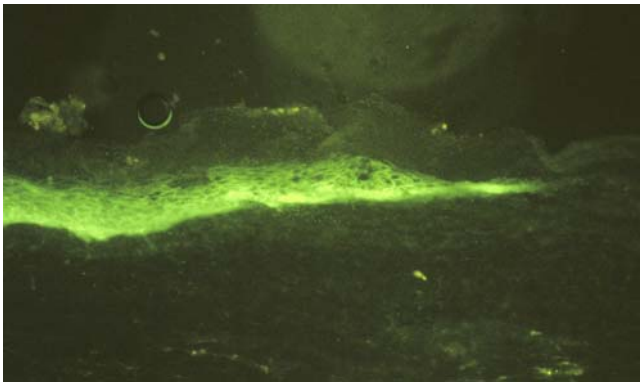

Fig. 8 MHC Class I Antigen detected by a FITC antibody proving the human origin of the epithelial cells, day 14 postoperatively after transplantation of keratinocytes seeded fibrin micro-carriers

The advantages of this new culture technique is the avoidance of any enzymatic treatment before transplantation compared to other culture methods [4, 7, 13]. Furthermore, the large culture surface allows for a broader attachment of viable cells. This was shown to lead to a higher yield of cells compared to standard culture techniques. The system is capable of reconstructing a multilayered, stable and regenerative epithelium in the immunocompetent porcine model; this is a verification of previous results in the immunoincompetent athymic nude mouse model [11, 12]. There was no cellular inflammatory reaction during the whole observation period

The epithelial thickness was comparable to KFGS-standard1-group after 14 days while the initial cell density was approximately 9-fold less and only one sixth of the cell amount of the associated mouse model.

In previous studies it has been shown that cells seeded on a carrier surface were able to reconstitute the epidermis like the suspension or sheet transplantation methods [6, 12]. The drawback was the inflammatory reaction of the organism to the carrier material. In this study we used a micro-carrier system consisting of fibrin avoiding foreign body reaction, but using the same benefit as the former micro-carrier systems [11, 12]. In addition one might transplant multiple cell types with this spinning culture system using fibrin micro-carriers which presents an interesting alternative for co-culture and co-transplantation in the field of tissue engineering. In addition to in vitro experiments, which showed promising results, we investigated the feasibility of fibrin carriers to be loaded with recombinant growth factor as a delivery system for rhEGF.

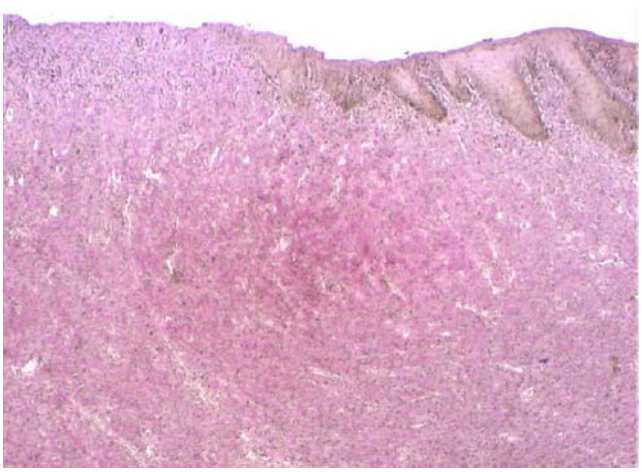

Fig. 7 Control wound without cells transplanted, HE staining of wound necropsy on day 21 postoperatively. There are no epithelium at the wound site, but ingrowth of mouse epithelium at the wound's edges

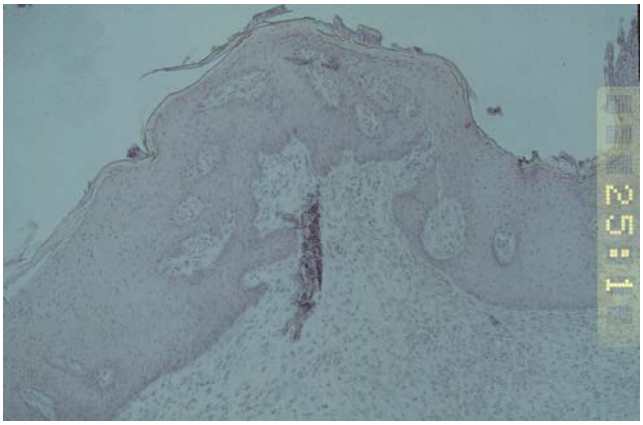

Fig. 9 Day 14 postoperatively after transplantation of autologous keratinocytes seeded fibrin micro-carriers in a pig animal model, formation of characteristic rete ridges, newly constructed dermal structures

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